

# Retinoic Acid Induced Differentiation and Commitment in HL-60 Cells

by Peter A. Meyer\* and Clemens Kleinschnitz\*

Human leukemic HL-60 cells are an established model for studies of differentiation induction. Retinoic acid (RA),  $2 \times 10^{-6}$ M, was used to induce terminal differentiation, assayed as nitroblue tetrazol reduction (NBT) and expression of monocytic surface antigens, which were detected by monoclonal antibody Leu M3. In addition, transferrin receptor expression and the number of S + G<sub>2</sub> + M-phase cells were determined. With a 12-hr RA incubation, only a decrease of transferrin receptor expression was found, with no change in other parameters. At least 96 hr RA incubation was necessary to induce terminal differentiation, with most cells being positive for NBT and M3. Cells induced with RA for 12 hr and subsequently recultured in liquid culture gradually expressed the differentiated phenotype and lost transferrin receptor expression. The number of S + G<sub>2</sub> + M-phase cells in the cultures decreased drastically. After 12 hr RA exposure and 120 hr reculture without RA, the differentiation profile was comparable to that of cells that had been induced with RA for 96 hr. In reculture for up to 120 hr there was no evidence of loss of viability or regrowth of possibly residual undifferentiated cells. From these studies, we conclude that HL-60 cells become committed to terminal differentiation after half a generation-time exposure to RA and remain committed for at least six generation times.

## Introduction

Cancer can be viewed as a disorder of gene expression resulting in uncoupling of the control of cellular proliferation and differentiation. In this context, neoplastic cell lines that can be induced to terminal differentiation can be used to study the modification of this process, possibly providing new options for the treatment of leukemias.

Normal hematopoietic cell development is depicted as proceeding from a morphologically immature stem cell with unlimited self-renewal capacity to progenitor cells committed to a specific lineage with limited self-renewal capacity and terminally differentiated lineage-specific cells with restricted or no self-renewal capacity. The strategy of differentiation induction in tumors is based on the assumption that by inducing differentiation, downregulation and eventually loss of self-renewal capacity of the malignant clone is achievable.

The human myeloid leukemic cell line HL-60 (1) is a bipotential cell that can be differentiated by various agents either to the monocytic or granulocytic pathway (2). Limited information is available on the question of how commitment to differentiation is achieved by induction of differentiation. Recent studies have addressed the question by separating early and late events

of differentiation, which are not necessarily coupled (3-5), and by using clonal assays after exposing cells to dimethylsulfoxide and retinoic acid (6,7). The present study takes a different experimental approach by using whole cell populations in liquid culture as the differentiation target. In addition to the NBT-assay, surface antigen expression of transferrin receptor (TfR) and Leu M3 (M3) were used to monitor cellular differentiation.

## Experimental Procedures

### Cell Line, Culture Conditions, and Viability

HL-60 cells were obtained from the American Tissue Culture Collection and routinely passaged at a density of  $2 \times 10^5$  cells/mL in RPMI 1640 (Seromed-Biochrom, Berlin, FRG), 10% (v/v) preselected fetal calf serum (FCS) (Gibco/BRL, Eggenstein, FRG), 100 U/mL penicillin G (Seromed) and 100 mg/L streptomycin (Seromed) in a 5% CO<sub>2</sub> automatically controlled atmosphere. The doubling time of the cells in the experiments was between 20 and 22 hr.

The cell suspension was incubated with 0.1% trypan blue (Sigma chemie, Deisenhofen, FRG) in 0.9% NaCl for 10 min. Trypan blue exclusion was determined by scoring 200 cells using a light microscope.

### NBT Assay

Using a commercially available nitroblue tetrazole (NBT) assay (Sigma), 0.1-mL cell suspension at a den-

\*Medizinische Poliklinik, University of Würzburg, D-8700 Würzburg, Federal Republic of Germany.

Address reprint requests to P. A. Meyer, Medizinische Poliklinik, University of Würzburg, D-8700 Würzburg, Federal Republic of Germany.

sity of  $10^6$  cells/mL were mixed with 0.2 mL NBT solution and 0.1 mL stimulant and incubated for 25 min at 37°C. Subsequently, cytocentrifuge slides were prepared. NBT reduction to blue-black formazan (NBT positive) after stimulation indicates oxidative bursts characteristic for mature granulocytic or monocytic cells. NBT-positive cells were determined by scoring 200 cells under a light microscope.

### S-Phase Assignment

The pellet obtained from washing  $10^6$  cells in RPMI 1640 was resuspended in 0.6 mL staining solution (0.1% Na-citrat, 50  $\mu$ g/mL propidium iodide, 0.1% Triton X-100, 0.05 mg/mL RNase) and incubated 30 min at 37°C. Relative DNA content was determined by measuring propidium iodide fluorescence at 380 nm using a FAC-Scan (Becton-Dickinson, Heidelberg, FRG), and cells were assigned to S + G<sub>2</sub> + M-phase according to relative DNA content.

### Leu M3 and Transferrin Receptor Expression

Monoclonal antibodies anti-Leu M3 fluorescein isothiocyanate conjugate (FITC) (Becton-Dickinson, specificity: human monocyte/macrophage antigen) and anti-transferrin receptor antibody FITC (Becton-Dickinson, specificity: human transferrin receptor) were used. After washing,  $10^6$  cells were resuspended in 50  $\mu$ L RPMI with 10% FCS and incubated with 20  $\mu$ L of the respective antibody for 30 min on ice. Cells were then washed with phosphate-buffered saline (PBS) resuspended in 500  $\mu$ L PBS, and the percentage of fluorescein-staining cells was measured using a FACScan. Controls for nonspecific staining were done using an irrelevant antibody, goat-anti-mouse (GAM-FITC, Becton-Dickinson).

### Experimental Design

HL-60 cells at a density of  $2.5 \times 10^5$  cells/mL were incubated in liquid culture with  $2 \times 10^{-6}$  M *cis*-retinoic acid (RA) for 12, 24, and 96 hr (incubation time). Controls were cultured without RA. Cells were removed from the culture and assayed for viability, scored for NBT reduction, and assayed for TfR expression, M3 expression, and DNA content. The remainder of the cells were washed three times in FCS-containing culture medium and reseeded in liquid culture at initial density without RA. At 24, 48, 72, 96, and 120 hr (reculture time), aliquots were removed from the cultures and cells assayed as described above. All experiments were done in triplicate; results are given as medians.

### Results

The present study using HL-60 cells addressed the question of commitment to differentiation after exposure to RA. In control cultures, 68 to 74% of cells were

TfR positive, 41 to 46% were in S + G<sub>2</sub> + M-phase, 0 to 0.8% were NBT positive, and 3 to 5% were M3 positive. Viability was 89 to 91%. No difference in these parameters was apparent after different times (0–120 hr) in liquid culture without RA.

### Reculture of HL-60 Cells after a 12-Hour RA Incubation

Cells were incubated with RA for 12 hr and recultured without RA for 24 to 120 hr (Fig. 1). Viability was 80 to 88%. After 24 hr reculture, there was a slight increase in NBT positive and M3 positive cells, and TfR positive cells had decreased to 7% (SD, 6.0). With longer reculture time, a progressive decrease of S + G<sub>2</sub> + M-phase cells to 12.75% (SD, 4.6) at 120 hr and an increase of differentiated cells with 64.5% (SD, 12.2) NBT positive and 58.6% (SD, 18.5) M3 positive cells was observed. Thus, after a 12-hr RA incubation and subsequent 96-hr reculture, cells displayed a phenotype comparable to cells incubated for 96 hr with RA. This suggests that after a 12-hr RA incubation, HL-60 cells are committed to differentiation and continue to differentiate without RA in the culture medium, comparable to cells incubated for 96 hr with RA.

### Reculture of HL-60 Cells after a 24-Hour RA Incubation

Reculture after a 24-hr RA incubation resulted in progressive differentiation with an increase of M3 positive cells to 52.0% (SD, 13.9) and NBT positive to 68.8% (SD, 13.6) of cells. TfR-positive cells remained low, between 1.7 and 4.6%. S + G<sub>2</sub> + M-phase cells started to decrease at 24 hr reculture time and reached 11.3% (SD, 4.8) at 120 hr.

### Reculture of HL-60 Cells after a 96-Hour RA Incubation

After a 72-hr reculture, viability had decreased significantly to 59.5% (SD, 21.9), and at 120 hr, viability had further decreased to 40.0% (SD, 17.0) (Fig. 2). The number of S + G<sub>2</sub> + M-phase cells remained low, between 6.5% (SD, 1.3) and 2.5% (SD, 1.7). NBT-positive cells remained virtually unchanged over the reculture time, between 81.3 and 88.7%. M3-positive cells increased gradually from 26.9% (SD, 8.8) to 69.5% (SD, 10.3).

### Discussion

The present study addressed the question of commitment to differentiation of a whole population of cells after removing the inducer in a time-dependent manner. The experimental design also allowed us to detect the regrowth of undifferentiated cells in a population for up to six generation times of the undifferentiated cells. The present study shows as a first event of RA-induced dif-

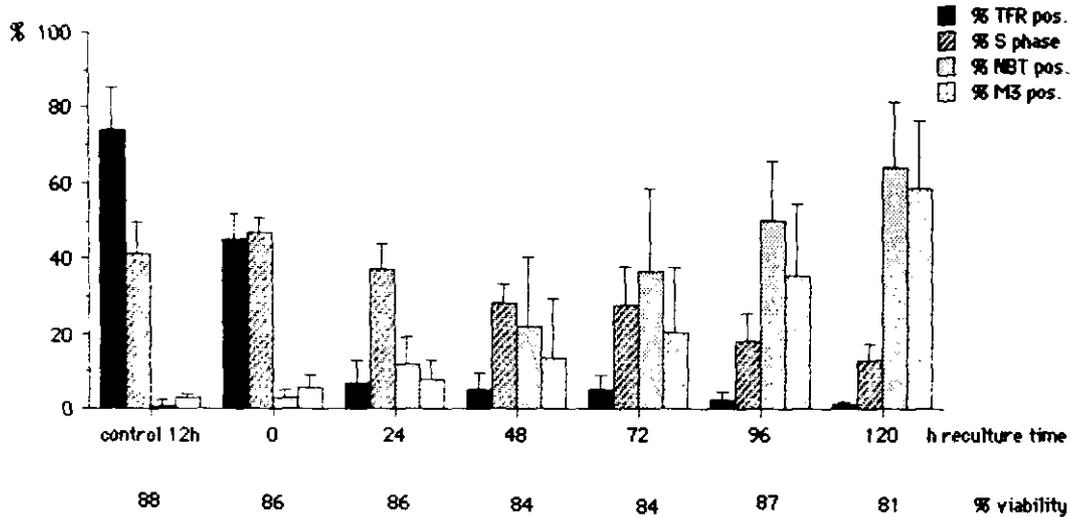


FIGURE 1. Kinetics of differentiation in HL-60 cells after a 12-hr incubation with  $2 \times 10^{-6}$  M RA. Cells were assayed for NBT reduction, TfR expression, M3 expression, trypan blue exclusion, and number of S + G<sub>2</sub> + M-phase cells after 24 to 120 hr reculture time without RA. Control 12 h denotes cells cultured for 12 hr without RA exposition. At 0 hr cells were assayed directly after 12 hr RA incubation.

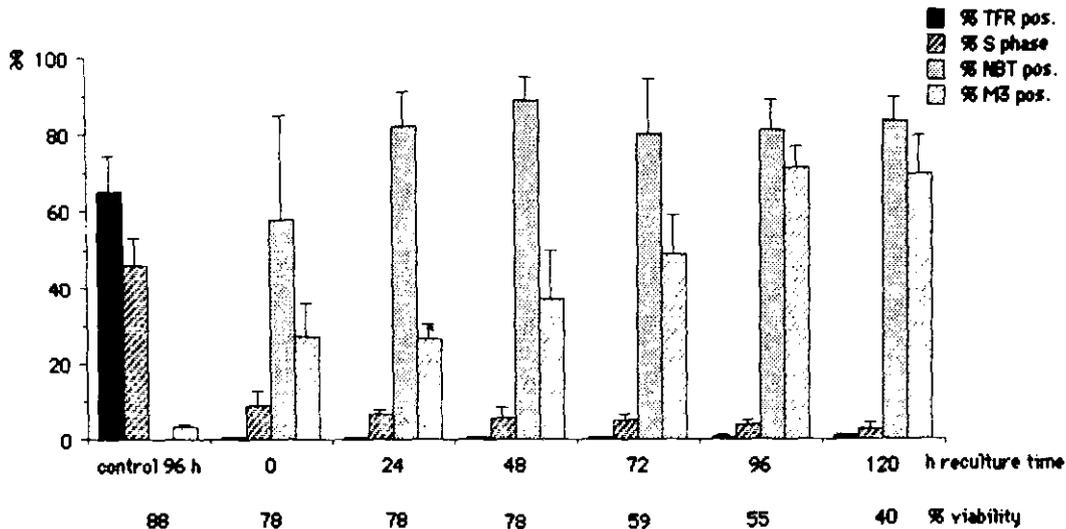


FIGURE 2. Kinetics of differentiation in HL-60 cells after a 96-hr incubation with  $2 \times 10^{-6}$  M RA. Cells were assayed for NBT reduction, TfR expression, M3 expression, trypan blue exclusion, and number of S + G<sub>2</sub> + M-phase cells after 24 to 120 hr reculture time without RA. Control 96 h denotes cells cultured for 96 hr without RA exposition. At 0 hr, cells were assayed directly after 96 hr RA incubation.

ferentiation a decrease in TfR expression, followed by a decrease in the number of S + G<sub>2</sub> + M-phase cells, and later by phenotypic maturation to NBT reduction and M3 expression. Viability is not affected for up to 96 hr of RA incubation, suggesting that cell death does not occur to a significant degree during this time interval. With reculture after 12 hr of RA incubation, the same series of events occurs. In addition, even after 120 hr of reculture, no regrowth of immature cells is detectable: the number of S + G<sub>2</sub> + M-phase cells and TfR-positive cells remains very low, suggesting self-propelled progression through the differentiation process. There is no evidence for regrowth of immature cells that could have escaped the effects of the inducer.

Phenotypic differentiation seems to be determined after 12 hr incubation with  $2 \times 10^{-6}$  M RA: cells need an additional 96 to 120 hr to proceed to terminal differentiation after termination of RA exposition. RA-mediated commitment to differentiation could be a result of altered gene transcription by RA. Alternatively, RA-mediated post-transcriptional effects or direct biochemical action of RA on cellular differentiation is possible.

Commitment of HL-60 cells to terminal differentiation by dimethylsulfoxide and RA has been reported using the NBT assay and clonal growth in plasma clots after cells had been exposed to the respective inducer in liquid culture (7). Evidence for commitment was that colonies became NBT positive and contained fewer cells

than control cultures. Data derived from sequential daughter cell transfer in semisolid medium, allowing determination of the fate of clones derived from one single cell (6), suggested that reversible losses of self-renewal capacity precede irreversible phenotypic differentiation. The limitation of the experimental approach in both cases is that it does not allow for detection of regrowth of relatively few cells in liquid culture after prolonged reculture without inducer. From both types of experiments, it was concluded that commitment to differentiation occurred in HL-60 cells. Further analysis using semisolid cultures in the present experimental setting is necessary to determine the correlation of changes in clonal growth with the parameters reported here. If leukemic cells *in vivo* can be committed to differentiation with differentiation inducers, a novel therapeutic modality for the treatment of leukemias becomes available.

This work was supported by the Deutsche Forschungsgemeinschaft, SFB 172, C3. Excellent technical assistance was provided by M. Haupt and B. Sommer.

## REFERENCES

1. Collins, S., Gallo, R. C., and Gallagher, R. E. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature* 270: 347-349 (1977).
2. Collins, S. J. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood* 70: 1233-1244 (1987).
3. Yen, A., Forbes, M., DeGala, G., and Fishbaugh, J. Control of HL-60 cell differentiation lineage specificity, a late event occurring after precommitment. *Cancer Res.* 47: 129-134 (1987).
4. Yen, A., Freeman, L., and Fishbaugh, J. Hydroxyurea induces precommitment during retinoic acid induced HL-60 terminal myeloid differentiation: possible involvement of gene amplification. *Leukemia Res.* 11: 63-71 (1987).
5. Yen, A., Powers, V., and Fishbaugh, J. Retinoic acid induced HL-60 myeloid differentiations: dependence of early and late events on isomeric structure. *Leukemia Res.* 10: 619-629 (1986).
6. von Melchner, H., and Hoeffken, K. Commitment to differentiation of human promyelocytic leukemia cells (HL-60): an all-or-none event preceded by reversible losses of self-renewal potential. *J. Cell. Physiol.* 125: 573-581 (1985).
7. Tsiftoglou, A. S., Wong, W., Hyman, R., Minden, M., and Robinson, S. H. Analysis of commitment of human leukemia HL-60 cells to terminal granulocytic maturation. *Cancer Res.* 45: 2334-2339 (1985).